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Neuronal Gene Expression Correlates of Parkinson's Disease with Dementia

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Abstract

Dementia is a common disabling complication in patients with Parkinson's disease (PD). The underlying molecular causes of Parkinson's disease with dementia (PDD) are poorly understood. To identify candidate genes and molecular pathways involved in PDD, we have performed whole genome expression profiling of susceptible cortical neuronal populations. Results show significant differences in expression of 162 genes ($P < 0.01$) between PD patients who are cognitively normal (PD-CogNL) and controls. In contrast, there were 556 genes ($P < 0.01$) significantly altered in PDD compared to either healthy controls or to PD-CogNL cases. These results are consistent with increased cortical pathology in PDD relative to PD-CogNL and identify underlying molecular changes associated with the increased pathology of PDD. Lastly, we have identified expression differences in 69 genes in PD cortical neurons that occur before the onset of dementia and that are exacerbated upon the development of dementia, suggesting that they may be relevant presymptomatic contributors to the onset of dementia in PD. These results provide new insights into the cortical molecular changes associated with PDD and provide a highly useful reference database for researchers interested in PDD.

Keywords

Parkinson's disease; gene expression; mRNA splicing; ; laser capture microdissection; dementia

Although predominantly considered a movement disorder, 30 to 70% of Parkinson's disease (PD) patients will develop associated dementia (PDD) and approximately 3 to 4% of all dementia is a result of PDD.^{1,2} PDD typically involves primary deficits in executive and visuo-spatial functions with secondary impairments in memory,³ resulting in significant reduction in quality of life.⁴

Pathologically, PD is characterized by protein aggregates, called Lewy bodies, in dopaminergic neurons of the substantia nigra. These Lewy bodies are composed of ubiquitinated α -synuclein and other proteins.⁵ PDD is associated with the spread of this Lewy body pathology into limbic and cortical areas.⁶⁻⁸ Although Alzheimer's disease (AD) pathology and Lewy body pathology

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frequently overlap in PDD, Lewy body pathology is associated with the dysexecutive and visuo-spatial dysfunction of PDD.^{9,10} In cortical layers V and VI, pyramidal neurons are particularly susceptible to Lewy body formation and cell death. Aggregation of proteins into LBs may injure neuronal cells, perhaps contributing to neurodegeneration¹¹⁻¹⁴; but, it is unclear which factors contribute to cortical neurodegeneration in PDD. Interestingly, a large family with identified α -synuclein locus triplication¹⁵ exhibits a clinical phenotype with high probability of dementia¹⁶ and has extensive cortical LBs and some glial cell cytoplasmic inclusions. However, aside from a handful of instances of LRRK2 mutations, none of the genes responsible for familial forms of PD have been shown to be mutated in the sporadic form of the disease, which constitutes >95% of individuals suffering from PD.

Numerous gene expression microarray studies have examined differential gene expression in the midbrain of PD patients. These studies have identified the altered expression of genes related to oxidative stress, inflammatory responses, protein degradation, vesicle trafficking, and protein chaperone functions.^{17,20} More recently, single cell profiling of dopaminergic neurons using laser capture microdissection identified alterations in signaling pathways, in genes involved in neuronal maturation, and in several protein kinases in the substantia nigra pars compacta (SNc) of PD patients.²¹ To date, there is no comprehensive study looking at gene expression in vulnerable cortical neurons of patients with PDD.

To probe the underlying molecular factors that contribute to cortical neurodegeneration in PDD, we have used laser-capture microdissection to isolate layer V-VI pyramidal neurons from the posterior cingulate cortex of 14 healthy control individuals, 15 cognitively normal PD patients (PD-CogNL), and 13 patients with PDD. We identify substantial alterations in cortical neuronal gene expression in PDD relative to either PD-CogNL or healthy controls, consistent with the onset of cortical pathology characteristic of PDD. In contrast, relatively few cortical genes are affected in PD-CogNL when compared with healthy controls. However, those genes that are dysregulated in PD-CogNL patients may provide insights into the underlying initiating events that lead to the subsequent development of dementia.

SUBJECTS AND METHODS

Tissue Collection

Posterior cingulate cortex samples were obtained from the Sun Health Research Institute Brain Bank. Samples included 14 individuals clinically and pathologically confirmed to be neurologically and cognitively normal (10 male, 4 female; age 78.6 ± 6.7 years), 15 PD-CogNL (11 male, 4 female; age 79.9 ± 6.5 years), and 14 PDD samples that were pathologically confirmed the absence of AD pathology (10 male, 4 female; age 75.5 ± 6.1 years). All cases signed informed consent at Sun Health Research Institute and were prospectively followed until death and autopsied according to previously published protocols.²² No case expired as a result of accident or suicide nor were kept alive heroically before death. Samples were selected with a postmortem interval less than 3 hours. Posterior cingulate cortex was sectioned at 8 μ m thickness and mounted onto standard, uncoated glass slides (Fisher Scientific). Slides were then stained with 1% neutral red, and pyramidal neurons were identified based on their characteristic size, shape, and location within layers V and VI. Approximately, 1,000 neurons were collected using the AutoPix (Arcturus) instrument. Total RNA was extracted using the Pico-Pure RNA isolation kit (Arcturus) following the manufacturer's protocol. DNase I treatment was performed as described in the manual.

RNA Amplification and Array Hybridization

All total RNA samples were double round amplified using Affymetrix's GeneChip Two-Cycle Target Labeling kit (Santa Clara, CA) with a T7 promoter and Ambion's MEGAscript T7 High

Yield Transcription kit (Austin, TX) as per manufacturer's protocol. Amplified and labeled cRNA was quantitated on a spectrophotometer and run on a 1% Tris-acetate EDTA gel to check for an evenly distributed range of transcript sizes. All samples were successfully amplified. Labeled cRNA (10 µg) was fragmented and hybridized to the Human Genome U133 plus 2.0 arrays following the standard protocols. Standard fluidics protocols were used to wash and stain the arrays (Euk genome WS2V5). Arrays were scanned using the GeneChip Scanner 3000 (Affymetrix).

Data Analysis

The Affymetrix Human Genome Arrays measure the expression of ~47,000 transcripts and variants, including 38,500 characterized human genes. All raw signal intensities were scaled using Affymetrix's Gene Chip Operating Software to a median signal intensity of 150 to enable interarray comparisons. Arrays included in the study passed stringent quality control metrics of at least 20% of genes expressed, a maximum 3'/5' GAPDH ratio of 30, and a scaling factor <10.

To identify significant gene dysregulation, any gene not expressed in at least two samples was first removed from the data set. Genes with average signal intensities less than 100 across all of the three groupings of control, PD-CogNL, and PDD were removed as differences in expression at these levels are likely to fall within the background for detection. Next heteroscedastic (two sample unequal variance), two-tailed t-tests were used to identify significant differences between PDD and control, PDD and PD-CogNL, and PD-CogNL and control. The entire data set is presented as Supplementary Table 3.

Quantitative, real-time reverse-transcriptase polymerase chain reaction. For this analysis, 500 layer V and VI pyramidal neurons were isolated from each of three independent posterior cingulate cortex samples from control, PD-CogNL, and PDD cases. RNA was isolated using the PicoPure RNA isolation kit (Arcturus) following the manufacturer's protocol. mRNA was reverse transcribed using SuperScript® II reverse transcriptase (Invitrogen) and oligo(dT). Following reverse transcription, the resulting cDNA was amplified by PCR with gene-specific primers that were generated using Primer3 software (<http://frodo.wi.mit.edu/>) and checked for specificity using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). PCR reactions were performed using the LightCycler (Roche), which allows real-time monitoring of the increase in PCR product concentration after every cycle based on the fluorescence of the dsDNA specific dye SYBR green.²³ The number of cycles required to produce a detectable product above background was measured for each sample. These cycle numbers were then used to calculate fold differences in starting mRNA level for each sample using the following method. First, the cycle number difference for a control gene, Histone 3B, was determined in the control sample and in the appropriate test sample. This difference was referred to as ΔH . Next, the cycle number difference for the gene of interest was determined in the control sample and in the appropriate test sample, yielding another value, ΔI . The cycle number difference for the gene of interest was then corrected for slight differences in the amount of total RNA in control and in drug treated samples by subtracting ΔH from ΔI , yielding a new value ΔK . The expression ratio for the gene of interest was then calculated as $2^{-(\Delta K)}$ for the genes that were induced, and as $-(2^{\Delta K})$ for the genes that were repressed. Specificity of each primer pair was confirmed by the melting curve analysis and agarose-gel electrophoresis. The expression ratios reported are the average of three replicate qRT-PCR reactions on RNA isolated from three independent samples. Statistical significance was calculated using a paired, two-tailed T-test.

RESULTS

A Subset of Cortical Neuronal Genes are Dysregulated in PD

To identify the underlying molecular changes associated with dementia in PD, we first determined the extent of posterior cingulate cortical neuronal gene dysregulation associated with either (PDD) or without dementia (PD-CogNL). There were 162 genes significantly altered in pyramidal neurons of PD-CogNL cortex when compared with controls ($P < 0.01$; supplementary Table 1). A subset of 21 of these genes with >2 -fold changes in expression are shown in Figure 1. These genes clearly distinguish PDD and PD-CogNL from matched controls and are thus dysregulated independently of dementia in PD. Neuronal cell processes affected include downregulation of the proteasome (PSMB4, -2.2 fold), decreased response to oxidative stress (OXR1, -3.0 fold), axonal transport (KIF21A, -2.1 fold), neurite outgrowth and axonal pathfinding (SLIT2, -2.0 fold), and synaptic transmission (NSAP1, $+2.0$ fold). There are also indications of altered neuron-glia interactions with the downregulation of FGF9 (-2.4 fold).

PD with Dementia is Associated with Increased Gene Dysregulation

To identify gene dysregulation associated with dementia in PD, we next compared gene expression in PDD samples to both PD-CogNL and to controls. Coincident with emerging cortical pathology in PDD, substantially more gene dysregulation (556 genes were altered in expression) was associated with dementia than previously identified as PD associated (Supplementary Table 2). The large number of genes associated with the onset of dementia suggests that the development of PDD symptoms and pathology evokes numerous secondary downstream effects on the physiology of the neuron. A subset of 73 of the differentially expressed genes, defined by significance at $P < 0.01$ and >2 -fold change in expression, is shown in Figure 2. Many of the same processes associated with PD remain altered in PD with dementia. In addition, genes involved in inflammation (SGPP2, $+3.4$ fold) and mitochondrial function become dysregulated in PDD (SSBP; -2.4 fold). The direction of change is consistent with increased inflammation and decreased mitochondrial function in PDD.

Identification of Gene Dysregulation Before Dementia Onset

Perhaps, highest interest is aberrant gene expression occurring before the onset of dementia and that increases in magnitude as dementia develops. These genes may provide the best candidate “initiators” of dementia. We identified genes that were significantly altered in a comparison of PD-CogNL to control ($P < 0.05$) and altered in the same direction in a comparison of PDD to PD-CogNL ($P < 0.05$). This set of genes are consistently altered across the continuum of disease progression from healthy control to PD-CogNL and then to PDD. This analysis identified 69 genes showing consistent and exacerbated gene dysregulation across the progression of disease from control to PD-CogNL and then to PDD (see Fig. 3).

Substantial alterations in pre-mRNA splicing machinery occur before dementia onset with the downregulation of SART3 (-1.4 fold), LUC7L (-1.5 fold), FBNP3 (-1.6 fold), PLRG1 (-1.3 fold), and FUS (-1.4 fold) in PD-CogNL versus controls (see Fig. 3). Furthermore, this downregulation increases in magnitude with disease progression, as evidenced by the further downregulation of these genes in a comparison of PDD to controls (SART3, -1.8 fold; LUC7L, -2.4 fold; FBNP3, -2.5 fold; PLRG1, -2.2 fold; and FUS, -1.9 fold). This finding suggests that alterations in mRNA splicing may be involved in early stages of progression to dementia in PD (see Discussion).

qRT-PCR Validation of Differential Gene Expression

To independently confirm the altered expression of several gene candidates, we performed qRT-PCR on mRNA from cortical neuronal cell populations isolated from the posterior cingulate cortex of three independent samples each of control, PD-CogNL, and PDD cases (see Methods). Individual genes chosen for validation were proteasome subunit, beta type 4 (PSMB4), slit homolog 2 (SLIT2), fibroblast growth factor 9 (FGF9), single-stranded DNA binding protein 1 (SSBP), sphingosine-1-phosphate phosphatase 2 (SGPP2), translocase of inner mitochondrial membrane 50 homolog (TIM50L), squamous cell carcinoma antigen recognized by T cells 3 (SART3), PRP40 pre-mRNA processing factor 40 homolog A (FNBP3), pleiotropic regulator 1 (PLRG1), Luc7 homolog-like (LUC7L), and heterogeneous nuclear ribonucleoprotein P2 (FUS).

Results confirmed statistically significant altered mRNA expression between PD-CogNL and controls for PSMB4 (−1.83 fold; $P = 0.016$) and SLIT2 (−2.45 fold; $P = 0.002$) (Fig. 4A). Results for FGF9 showed a trend toward downregulation that did not reach statistical significance (−1.36 fold, $P = 0.103$), and TIM50L showed no change (1.07 fold, $P = 0.53$). For the comparison of PDD to controls, qRT-PCR measurements showed both SGPP2 upregulation (+2.97 fold, $P = 0.008$) and SSBP downregulation (−2.74 fold, $P = 0.002$) (Fig. 4A).

Results for the selected genes across all three sample populations are shown in Figure 4B. These results confirm significant downregulation of FNBP3 (−1.46 fold, $P = 0.024$) in PD-CogNL cortical neurons when compared with controls. Several additional transcripts coding for proteins involved in the pre-mRNA splicing machinery trended toward downregulation in this comparison (SART3, −1.21 fold, $P = 0.092$; PLRG1, −1.31 fold, $P = 0.17$; and FUS, −1.23 fold, $P = 0.075$). Only PLRG1 showed statistically significant downregulation in the comparison of PDD to PD-CogNL (−1.82 fold, $P = 0.012$). However, both FNBP3 (−1.35 fold, $P = 0.089$) and LUC7L (−1.61 fold, $P = 0.069$) showed a strong trend toward downregulation. Each of the genes tested showed statistically significant downregulation in a comparison of PDD neurons to control neurons (SART3, −2.17, $P = 0.009$; FNBP3, −2.08 fold, $P = 0.013$; PLRG1, −3.11 fold, $P = 0.001$; LUC7L, −1.62, $P = 0.048$; and FUS, −1.96 fold, $P = 0.014$). These findings confirm downregulation of certain members of the pre-mRNA splicing machinery in PDD cortical neurons and suggest that this downregulation may occur before dementia onset. This suggests the possibility that underlying splicing defects may be involved in the disease pathogenesis.

DISCUSSION

PDD is Associated with Substantial Cortical Gene Dysregulation

Analyses comparing cortical neuronal gene expression differences identified the largest number of significant differences in PDD neurons versus control neurons. This contrasted sharply with the relatively few changes found in PD-CogNL neurons (see Fig. 1). This is perhaps not surprising because cortical pathology, and presumably neuronal dysfunction, is substantially greater in PDD than in PD-CogNL. However, one would expect that gene expression changes that drive the clinical symptoms of dementia should occur before the onset of dementia, although the precise temporal relationship of specific gene dysregulation to the timing of symptom onset is unknown.

To identify the genes most likely to contribute to dementia in PD, we queried the data set for genes that were similarly affected in both PD-CogNL versus controls and in PDD versus PD-CogNL. The 69 differentially expressed genes showing consistent and significant changes across the continuum of disease states that were identified in this analysis function in processes have previously been heavily implicated in PD and, more generally, in neurodegeneration.

These include axonal transport, neurite outgrowth, cell adhesion, synaptic transmission, oxidative stress, and proteasome function. Of these, axonal transport, cell adhesion, and mRNA splicing appear to be major themes of dysregulation that occur before dementia onset (see Fig. 3).

From the analysis in Figure 3, axonal dysfunction emerges as a major theme of dysregulation occurring before and during the development of dementia in PD. Expression of microtubule motor proteins KIF21A (−3.4 fold), D2LIC (−2.2 fold), and KIF5A (+2.2 fold) and the tubulin chaperone TBCA (−1.6 fold) are all altered. In addition, genes involved in neurite outgrowth (FEZ20, −1.6 fold; LAMB1, −2.2 fold) are affected, as are genes involved in cell adhesion (Vezatin, −1.7 fold). These findings imply that defects in synaptic transmission and axonal function are early events in the pathogenesis of PDD.

Alterations in Expression of mRNA Splicing Components

The downregulation of numerous genes involved in pre-mRNA splicing is intriguing in light of recent studies that have shown that mitochondrial damage induced by paraquat alters splicing of parkin mRNA in a neuroblastoma cell culture model.²⁴ In addition, in vivo alterations in expression of parkin splice variants in sporadic PD²⁵ and in dementia with Lewy bodies (DLB)²⁶ have been demonstrated, suggesting that there may be underlying problems with mRNA splicing in PD. For these reasons we focused our qRT-PCR efforts on genes involved in pre-mRNA splicing. Our findings indicate significant downregulation of multiple components of the splicing machinery (Fig. 4B). These results raise the possibility that decreased expression of specific components of the mRNA splicing machinery may underlie differential splice variants associated with PDD. Further, these results may provide a possible practical rationale for linking mitochondrial dysfunction to the altered expression of multiple alternatively spliced transcripts through a yet-to-be-identified feedback loop wherein malfunctioning mitochondria could lead to altered expression of splicing components and subsequent deficits in alternative splicing.

In summary, this study represents a comprehensive expression profiling data set detailing the gene expression changes in cingulate cortical neurons of individuals with PD and PDD. This data set provides a starting point for more detailed mechanistic studies to identify the molecular etiology of cortical degeneration in PDD and will provide a valuable reference for researchers studying PDD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

A unique set of dysregulated genes in cortical neurons are associated with Parkinson's disease. Shown are heat maps representing the expression levels of each gene (horizontal rows) in all control, PD-CogNL, and PDD samples (vertical columns). Red indicates significant gene induction associated with PD and blue indicates significant repression. Heat maps were generated using GeneCluster version 2.1.7. Text columns represent (from left to right) the Affymetrix Probe ID, Gene Symbol, fold change and *P*-value for PD-CogNL vs control, fold change and *P*-value for PDD vs PD-CogNL, and fold change and *P*-value for PDD vs control. Only genes with at least a 2-fold increase or decrease are shown. Genes are ordered based on their fold change in the PD-CogNL vs control.

FIG. 2.

A unique set of cortical neuronal genes are dysregulated in Parkinson's disease with dementia. Shown are heat maps representing the expression levels of each gene (horizontal rows) in all control, PD-CogNL, and PDD samples (vertical columns). Red indicates significant gene induction associated with PD and blue indicates significant repression. Heat maps were generated using GeneCluster version 2.1.7. Text columns represent (from left to right) the Affymetrix Probe ID, Gene Symbol, fold change and *P*-value for PD-CogNL vs control, fold change and *P*-value for PDD vs PD-CogNL, and fold change and *P*-value for PDD vs control. Only genes with at least a 2-fold increase or decrease are shown and are ordered based on their fold change in the PDD vs PD-CogNL comparison.

FIG. 3.

A subset of genes may underlie the onset or progression of dementia in PD. Shown are all genes with altered expression occurring before the onset of dementia and that are further altered following the development of dementia associated with PD. All column labels are as for figures 2 and 3. Genes were ordered based on their fold change in PD-CogNL vs controls.

FIG. 4. qRT-PCR experiments confirm significant gene dysregulation in PDD and PD-CogNL cortical neurons. Shown in (A) are results for the genes indicated on the X-axis. The relevant comparisons are indicated across the top of the graph. A single asterisk indicates significance at the $P < 0.05$ level. Double asterisks indicate significance at the $P < 0.01$ level. Specific fold changes are reported in the text. Shown in (B) are results for the indicated genes and comparisons. Asterisks have the same significance as in A and specific fold changes are reported in the text.